Cell

DRAM, a p53-Induced Modulator of Autophagy, Is Critical for Apoptosis

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SUMMARY

Inactivation of cell death is a major step in tumor development, and p53, a tumor suppressor frequently mutated in cancer, is a critical mediator of cell death. While a role for p53 in apoptosis is well established, direct links to other pathways controlling cell death are unknown. Here we describe *DRAM* (*d*amage-*r*egulated *a*utophagy *m*odulator), a p53 target gene encoding a lysosomal protein that induces macroautophagy, as an effector of p53-mediated death. We show that p53 induces autophagy in a DRAM-dependent manner and, while overexpression of DRAM alone causes minimal cell death, DRAM is essential for p53-mediated apoptosis. Moreover, analysis of *DRAM* in primary tumors revealed frequent decreased expression often accompanied by retention of wild-type p53. Collectively therefore, these studies not only report a stress-induced regulator of autophagy but also highlight the relationship of DRAM and autophagy to p53 function and damage-induced programmed cell death.

INTRODUCTION

Inactivation of cell-death pathways is a central component of cancer progression (Hanahan and Weinberg, 2000). Various mechanisms exist in normal human cells to invoke cell death and eradicate damaged cells that may otherwise multiply and form a tumor (Crighton and Ryan, 2004). Consequently a number of known death regulators are mutated or lost in cancer. In particular, the *p53* tumor suppressor, a potent inducer of apoptotic cell death, is mutated in approximately 50% of all tumors (Beroud and Soussi, 2003).

The induction of cell death by p53 occurs via both target gene activation and transactivation-independent mechanisms at mitochondria (Moll and Zaika, 2001). In response to various forms of cellular stress, the levels of p53 increase and, after rapid localization of a proportion of p53 to mitochondria (Erster et al., 2004), p53 accumulates in the nucleus where it transactivates a number of proapoptotic target genes (Crighton and Ryan, 2004).

Apoptosis is an evolutionarily conserved, orchestrated cell-death process characterized by membrane-blebbing, DNA fragmentation, and the formation of distinct apoptotic bodies that contain components of the dead cell (Edinger and Thompson, 2004). This process occurs without membrane breakdown and does not elicit an inflammatory response, with apoptotic bodies being eventually removed by phagocytic cells. Central to this apoptotic process are a group of cysteine aspartyl proteases or caspases, which effect the destruction of the cell in an orderly fashion.

Autophagy (strictly the form termed macroautophagy, but hereafter for simplicity referred to as autophagy) is an evolutionarily conserved membrane-trafficking process that operates at basal levels under normal conditions as a means of degrading cytosolic proteins and organelles. Cytosol and organelles such as mitochondria and endoplasmic reticulum are engulfed into double-membraned vesicles called autophagosomes (induction step). Fusion subsequently occurs between the autophagosomes and lysosomes to form autolysosomes in which the cargo of the autophagosome is degraded by lysosomal hydrolases (turnover step) (Baehrecke, 2005). Autophagy is induced above basal levels in response to diverse stimuli including nutrient starvation (or trophic factor withdrawal that leads to starvation), genotoxic agents, phorbol ester with zVAD-fmk, or cytokines (Boya et al., 2005; Kuma et al., 2004; Lum et al., 2005; Mills et al., 2004; Shimizu et al., 2004; Yu et al., 2004). Numerous reports have implicated induction of autophagy in controlling cell viability. In response to nutrient deprivation or trophic factor withdrawal, autophagy is induced to sustain metabolism by producing

catabolites through the targeted proteolysis of long-lived proteins. In this context, therefore, autophagy acts as a self-limited survival mechanism (Kuma et al., 2004; Lum et al., 2005). Programmed cell death can also be triggered by nutrient deprivation, and autophagy induced in this context can serve to counter the induction of the cell-death program (Boya et al., 2005). Presumably, in this context, autophagy again acts to maintain pools of cellular metabolites to balance the lack of exogenous nutrients. However, autophagosome accumulation is frequently observed within cells undergoing programmed cell death, for example, in cells treated with tumor necrosis factor-related apoptosis-inducing ligand, or in hormone-induced cell death in the *Drosophila* salivary gland. This has led to the contrary suggestion that autophagy may promote cell death, with the selective removal of survival factors or prolonged removal of cellular constituents resulting in the demise of the cell (Gozuacik and Kimchi, 2004; Lee and Baehrecke, 2001; Mills et al., 2004; Ogier-Denis and Codogno, 2003).

Importantly, several recent studies have now explicitly assessed autophagy as a potential contributor to programmed cell death. In situations where pro-death stimuli such as genotoxic agents, staurosporine, or phorbol ester are administered to cells, in which proteins that normally effect apoptotic death are either deleted or inhibited, a caspase-independent programmed cell death dependent on autophagy occurs (Shimizu et al., 2004; Yu et al., 2004). However, it is unclear how the induction of autophagy downstream of pro-death stimuli affects classical apoptotic caspase-dependent cell death in a physiological context where components of this pathway are not experimentally compromised. It has, however, been suggested that autophagy may promote apoptotic cell death during NGF withdrawal from sympathetic neurons (Xue et al., 1999). However, the generality of this observation is yet to be determined. Furthermore, it has been reported that antiapoptotic Bcl-2 family members can also modulate autophagy (Pattingre et al., 2005; Shimizu et al., 2004). In the context of nutrient stress, Bcl-2-mediated inhibition of autophagy, via interaction with the autophagy mediator Beclin1, is reduced allowing cells to respond to metabolic stress through induction of autophagy (Pattingre et al., 2005). However, it has also been suggested that Bcl-2 family members (Bcl-2/Bcl-x_L), presumably through an alternate mechanism, can promote the autophagy that is a component of nonapoptotic programmed cell death seen in response to staurosporine or etoposide when their binding partners (Bax/Bak) are experimentally removed (Shimizu et al., 2004). It is not known, however, whether Bcl-2 affects autophagy during apoptosis. Overall the question appears to be not whether autophagy per se causes cell death or promotes cell survival, but how different stimuli induce autophagy as a context-specific mediator of cell death or cell survival. For example, why might pro-death stimuli promote autophagy such that this contributes to programmed cell death, whereas under starvation conditions, autophagy is induced to maintain viability? Recent work may suggest that in response to different stimuli, autophagy may act to selectively target different protein or organelle cargos. For example, under starvation conditions clearly the nonselective turnover of long-lived proteins would be sufficient to provide catabolites for energy production. In other situations, certain proteins may be specifically targeted for proteolysis. For example, during the caspase-independent cell death dependent on autophagy, catalase may be specifically degraded. In contrast, catalase is not selectively removed under nutrient-deprived conditions (Yu et al., 2006). Observations of selective turnover may ultimately resolve the paradoxical roles of autophagy in different contexts.

While autophagy may function in different contexts to either promote or inhibit cell survival, downstream of diverse stimuli, the signaling pathways regulating these diverse forms of autophagy remain poorly defined. Clearly therefore, the identification of further regulatory factors that signal induction of autophagy, the specific contexts in which these are important, and how the induced autophagy affects cell viability is required to fully understand cell-death regulation and tumor suppression. We report here the identification of a novel stress-induced regulator of autophagy that we have termed *DRAM* for *d*amage-*r*egulated *a*utophagy *m*odulator. *DRAM* is a direct target of p53 and, while p53 has previously been shown to modulate autophagy (Feng et al., 2005), we show that p53 induces autophagy in a DRAM-dependent manner. Furthermore, we show that DRAM is critical for p53-induced cell death and that DRAM is downregulated in human cancer.

RESULTS

Identification of DRAM

To identify novel components of p53′s cell-death response, we used a Saos-2 cell line (null for endogenous p53) that contains a doxycycline (Dox)-inducible *p53* transgene (TetOn-p53) (Ryan et al., 2000). Based on the expression profiles of two known p53 target genes, *p21* and *PUMA* (el-Deiry et al., 1993; Nakano and Vousden, 2001; Yu et al., 2001), we examined mRNA changes 24 hr after p53 induction (data not shown). Microarray analysis revealed a number of mRNA species responsive to p53 over this timeframe. Amongst these, the only previously uncharacterized mRNA that exhibited induction comparable to known p53 targets encodes the hypothetical protein FLJ11259 (accession number BC018435)—referred to here as *DRAM*.

Human *DRAM* encodes for a polypeptide of 238 amino acids (Figure 1). Analysis of this peptide sequence indicated that DRAM contains a putative signal peptide for targeting to the endoplasmic reticulum (ER) and six hydrophobic potential transmembrane regions (Figure 1A). DRAM is highly conserved in a number of species including mouse, zebrafish, *Drosophila*, and *C. elegans*. No orthologs were found, however, in simpler organisms such

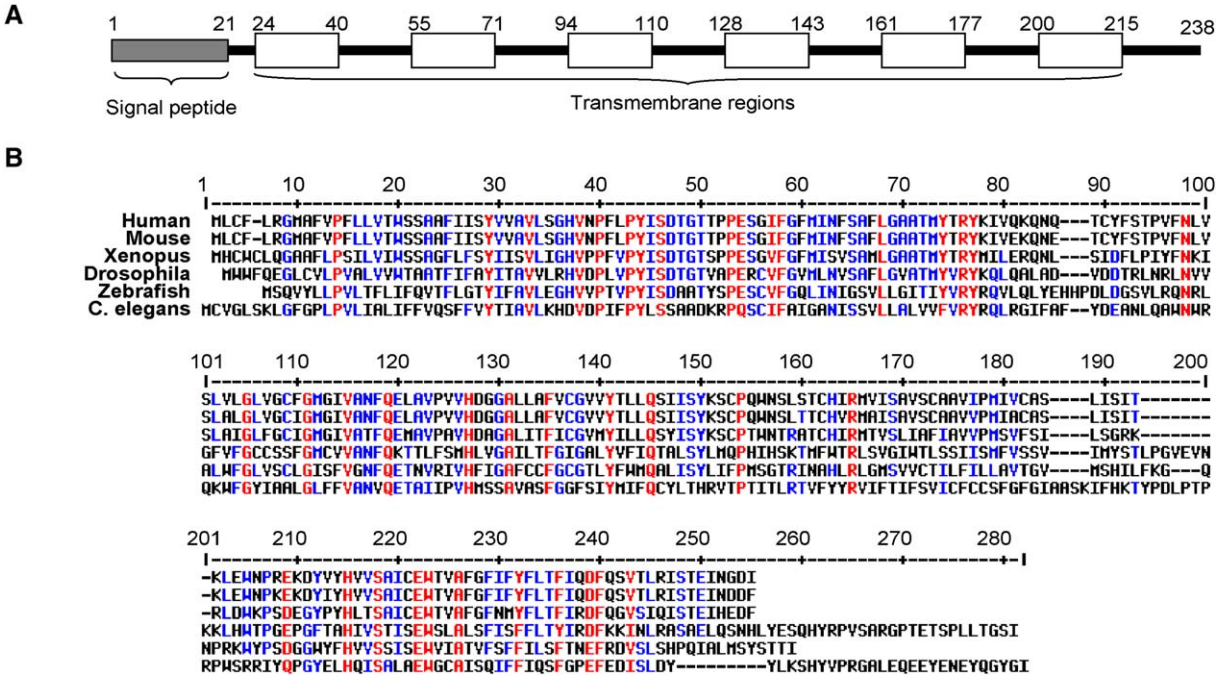

Figure 1. DRAM Is an Evolutionarily Conserved Protein with Hydrophobic Domains and a Predicted ER Signal Peptide

(A) Human DRAM consists of 238 amino acids. Domain predictions indicate six hydrophobic transmembrane regions (open boxes) and an ER signal peptide (gray).

(B) Alignment of DRAM from various species. High amino acid conservation (red), low amino acid conservation (blue) are shown.

as yeast or bacteria. Alignment of these DRAM sequences exemplifies this conservation and reveals specific domains and residues that are conserved (Figure 1B).

Regulation of DRAM by p53 and DNA Damage

To confirm the induction of *DRAM* seen in the microarray analysis, mRNA from TetOn-p53 cells treated with Dox for 24 hr was analyzed by semiquantitative RT-PCR and real-time quantitative RT-PCR (qPCR). In these cells, *DRAM* was found to be induced 8-fold (Figures 2A and 2B). *DRAM* was also induced marginally by the transactivation-impaired p53 mutant 175H (Figures 2A and 2B). However, a similar induction was also observed for the well-characterized p53 target gene, *p21* (Figure S1A). *DRAM* was also induced by p53 in another cell system containing a p53-ER fusion protein (p53 fused to the hormone binding domain of the estrogen receptor) (Figure 2C) that is responsive to tamoxifen (Tam) (Littlewood et al., 1995). RNA species that are induced by Tam even in the presence of protein synthesis inhibitors (e.g., cycloheximide [CHX]) do not require the synthesis of an intermediary protein and can therefore be considered primary targets of p53. Similar to the activation observed for *p21* (Figure S1B), *DRAM* was induced by p53-ER by approximately 3.5-fold. This induction was also evident in the presence of CHX, indicating that p53-mediated induction of *DRAM* is a direct effect (Figure 2C). The levels of *DRAM* were also increased by the addition of CHX alone. This has previously been shown for a number of mRNA species (a similar effect was seen for *p21* mRNA) (Figure S1B) and is considered to indicate that the levels of these transcripts are under the control of either a short-lived transcriptional repressor or a short-lived factor that causes mRNA destabilization (Grandori et al., 1996; Wilson and Freeman, 1996). Nevertheless, the further induction of *DRAM* by p53 in the presence of CHX indicates that this is a direct effect.

Next we tested if *DRAM* was induced by cellular stresses and if these effects were dependent on activation of endogenous p53. p53 wild-type RKO cells stably expressing either a short hairpin RNA (shRNA) to inactivate p53 (pRS-p53) or a nonsilencing shRNA (pRS-Scr) control were treated with the genotoxic agents actinomycin D (Act D), adriamycin, and etoposide. In each case *DRAM* was dramatically induced in control cells but not in those where p53 was silenced (Figures 2D and 2E)—demonstrating a strong dependency on p53 for induction. Moreover, this dependency was comparable to or greater than that seen for *p21* and *PUMA* (Figures S2A and S2B). Similar effects were also seen when p53 was inactivated by the E6 protein from human papilloma virus (data not shown). These data, taken together, indicate that *DRAM* is induced by cellular stresses via endogenous p53.

Since the p53-mediated induction of *DRAM* mRNA could not be explained by changes in *DRAM* mRNA stability (Figure 2F), we sought to determine if *DRAM* was a direct transcriptional target of p53. We searched the *DRAM* gene for consensus p53 binding sites using the p53MH

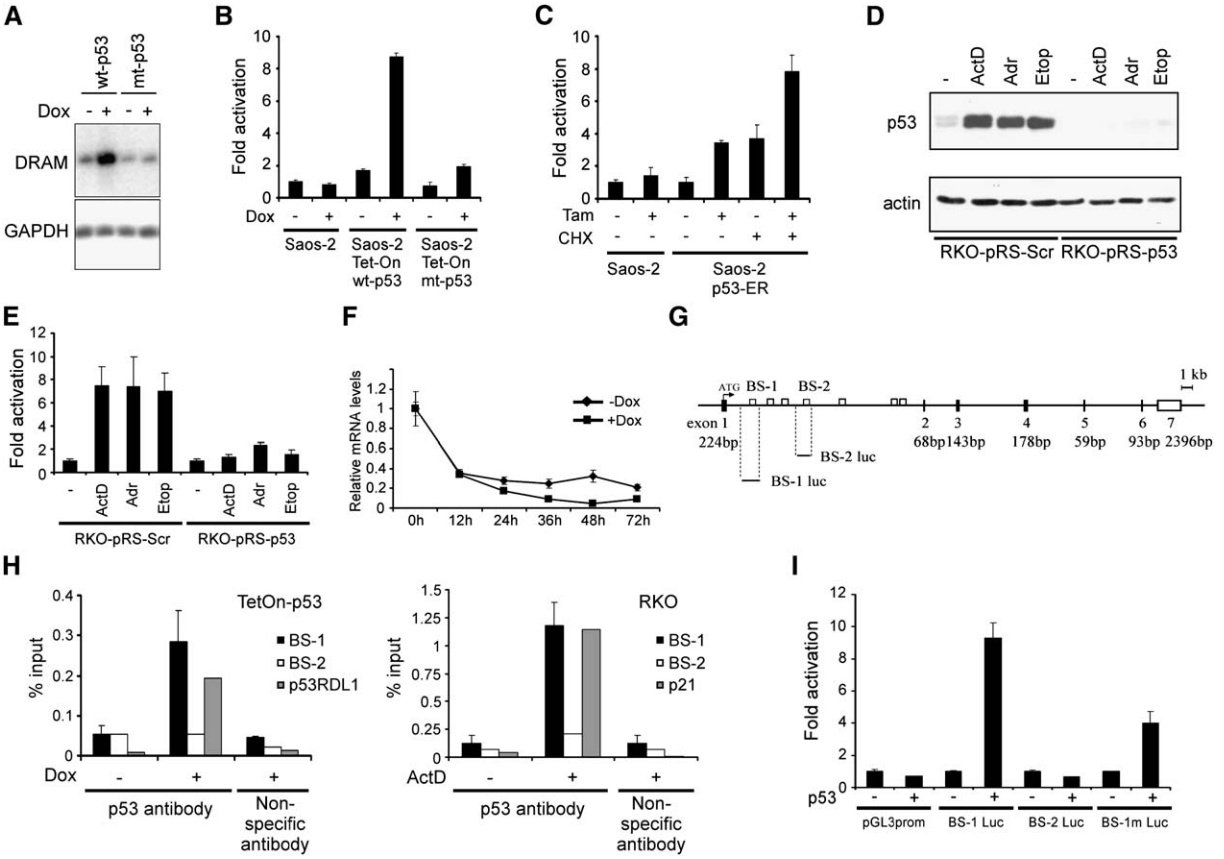

Figure 2. *DRAM* Is Induced by DNA Damage and Is a Direct Target of p53

DRAM is induced by p53. (A and B) Saos-2, TetOn-p53, or TetOn-p53(175H) cells were treated with Dox for 24 hr and mRNA levels assayed by semi-quantitative RT-PCR (A) and qPCR (B).

(C) *DRAM* is a direct target gene of p53. Saos-2 cells, parental or expressing a p53-ER fusion protein, were treated with 100 nM tamoxifen (Tam) and/or 10 μg/ml CHX for 24 hr. Levels of mRNA were analyzed by qPCR.

(D and E) *DRAM* is induced by genotoxic stress in a p53-dependent manner. RKO-pRS-Scr and RKO-pRS-p53 cells were treated for 12 hr with 1 nM actinomycin D (ActD), 0.06 μg/ml adriamycin (Adr), or 20 μM etoposide (Etop). (D) Levels of p53 were analyzed by Western blotting (D) and mRNA levels of *DRAM* were analyzed by qPCR (E).

(B), (C), and (E) are presented as mean fold activation ± SEM.

(F) Stability of *DRAM* mRNA is not altered by p53. TetOn-p53 cells were incubated with Dox for 12 hr followed by treatment with 10 μg/ml α-amanitin. At the indicated time points, levels of *DRAM* mRNA were analyzed by qPCR and presented as mean ± SEM.

(G) Schematic representation of the genomic organization of *DRAM*. Exons are shown as filled boxes with sizes indicated. The majority of exon 7 is noncoding. Potential p53 binding sites in intron 1 are shown as raised boxes above the line. BS-1 and BS-2 are indicated.

(H and I) p53 binds to and activates BS-1. (H) Chromatin immunoprecipiation was performed on TetOn-p53 cells treated with Dox (24 hr) or ActD-treated RKO cells (12 hr). Immunoprecipitations were carried out with anti-sera against p53 or a nonspecific antibody. The % input of coprecipitating DNAs were calculated by qPCR and presented as mean ± SEM. (I) BS-1 is responsive to p53. Luciferase reporter constructs containing BS-1, BS-2, or a construct where BS-1 had been mutated (BS-1m Luc) were assayed for transactivation by wild-type p53 in Saos-2 cells 24 hr after transfection with the indicated reporters and either p53 or vector control, pGL3prom. Data are represented as mean fold activation ± SEM.

algorithm (Hoh et al., 2002). A number of potential binding sites were identified within the first intron (Figure 2G). Analysis of a number of these sites by chromatin immunoprecipitation (ChIP) from TetOn-p53 cells and RKO cells revealed a potential binding site approximately 2.3 kb from the end of exon 1. This site, BS-1, was effectively immunoprecipitated by p53-specific antisera but not by nonspecific sera, as was also seen for established p53 response elements from known target genes (Figure 2H) (Tanikawa et al., 2003). Another potential binding site, BS-2, although having similar predictions of likely responsiveness as BS-1, was not specifically immunoprecipitated by antisera to p53 (Figure 2H).

To test whether the *DRAM* p53 binding site BS-1 was responsive to p53, a 765 bp region of *DRAM* intron 1 containing this site was cloned into a luciferase reporter plasmid. In addition, a reporter construct was also generated containing a 1478 bp region of the intron around the BS-2 site. Transfection of these reporter constructs into Saos-2 cells, together with a p53 expression plasmid,

revealed that the reporter containing BS-1, but not the one containing BS-2, was responsive to p53 (Figure 2I). Moreover, in accordance with mutational analysis of the p53-responsive element in the *PUMA* promoter (Yu et al., 2001), mutation of the BS-1 site in this reporter construct caused a marked reduction in the responsiveness to p53, proving that BS-1 is a p53-responsive element (Figure 2I). Mutation of this site, however, did not completely inactivate the responsiveness of the plasmid, indicating that either the mutation did not completely inactivate p53 binding or that this region of the intron also contains other p53-responsive elements.

Involvement of DRAM in Cell Death from p53

Since our data indicate that *DRAM* is a new direct target of p53, we assessed if *DRAM* induction contributes to p53′s apoptotic response. Two *DRAM*-specific siRNAs were generated that cause considerable knockdown of *DRAM* expression while not affecting the induction of p53, p21, and *PUMA* in response to Dox in TetOn-p53 cells or ActD in RKO cells—in which two-thirds of the death is dependent on p53 (Ryan et al., 2000) (Figures 3A, 3B, 3D, 3E and S3). However, both of these siRNAs caused a dramatic decrease in the amount of cell death observed, when compared to cells transfected with nonsilencing siRNA control (Figures 3C and 3F). Moreover, this decrease in death following DRAM knockdown was reflected in terms of long-term survival as it was found to cause a considerable increase in the clonogenic potential of RKO cells following transient ActD treatment (Figure 3G).

These findings indicated a major role for DRAM in p53-mediated cell death. We therefore assessed whether *DRAM* was able to induce death when expressed alone. Firstly, we overexpressed *DRAM* by transient transfection in Saos-2 cells where there are no potential other signals from p53. In contrast to the clear role of DRAM in p53-mediated cell death (Figures 3C, 3F, and 3G), *DRAM* induced very little death in this assay (approximately 2%–3%, Figure 4A) despite confirmation of expression by Western blotting (Figure 4B). However, cell death from *DRAM* when expressed alone may occur slowly and may not be apparent in transient transfection assays. Therefore, we assayed the ability of DRAM to affect clonogenic survival when expressed continually over a period of time. Saos-2 cells were transfected with selectable expression constructs for wild-type *p53*, mutant *p53*, and *DRAM*. Following selection, cells were assayed for the effects of the transfected plasmids on clonogenicity. Consistent with previous reports, transfection of *p53*, when compared with cells transfected with mutant *p53* or vector alone, caused a dramatic reduction in the number of colonies (Figure 4C). In contrast, but consistent with transient cell-death assays (Figure 4A), expression of *DRAM* did not alter colony formation (Figure 4C) despite continued DRAM expression in pools of selected colonies (Figure 4D). Taken together, our data suggest that DRAM is necessary but not sufficient for cell death from p53.

DRAM Is a Lysosomal Protein that Regulates Autophagy

To examine the function of DRAM further in whole populations of cells and in a temporally controlled manner, we generated a Dox-regulated cell line in p53 null Saos-2 cells that undergoes strong induction of DRAM following treatment with Dox (Figure 5A). Consistent with the data obtained from our death assays (Figures 4A and 4C), DRAM induction causes only a small increase in the number of cells with a sub-G1 DNA content (from 2% to 5% at 24 hr; data not shown).

We used these DRAM-inducible cells to determine the subcellular localization of DRAM. Since topology predictions indicated an ER-signal peptide and transmembrane regions (Figure 1A), we would expect DRAM to be localized in the membrane of a compartment of the secretory pathway. Staining for DRAM showed no localization at the endoplasmic reticulum, plasma membrane, Golgi apparatus, or the early endosome (data not shown). However, clear colocalization of DRAM was seen when cells were stained with an antibody for cathepsin D, either in the absence or presence of p53, suggesting that DRAM localizes to lysosomes (Figure 5B). Although analysis of endogenous DRAM would be required to confirm this localization, transient transfection of *DRAM* into Saos-2 and other cell types also revealed a staining pattern coincident with lysosomes (data not shown).

Due to the lysosomal localization of DRAM we postulated that DRAM may be regulating cell death in one of two ways. Initially, we considered that DRAM may mediate lysosomal membrane permeabilization causing release of proteolytic enzymes (cathepsins), but inhibitors of cathepsins (zFA-fmk and CA-074) did not affect the small amount of cell death seen following induction of DRAM in TetOn-DRAM cells, despite inhibition of this death with the caspase inhibitor, zVAD-fmk (data not shown). We next assessed, due to an integral role of lysosomes in autophagy, whether DRAM and also p53 (since *DRAM* is a direct p53 target) regulate autophagy. Analysis by electron microscopy revealed an accumulation of double-membraned autophagic vesicles following induction of either DRAM or p53 (Figures 5C and 5D). Consequently, we next looked for changes in the distribution of the autophagy marker LC3 (Kabeya et al., 2000). The bulk of LC3 exists in a form, LC3-I, which exhibits diffuse staining within the cytoplasm. When autophagosomes form, LC3-I is lipid conjugated to form LC3-II and is associated with the membrane of autophagosomes. Under these conditions, LC3 is visualized in small puncta corresponding to autophagosomes (Kabeya et al., 2000). TetOn-DRAM and TetOn-p53 were infected with an adenovirus expressing LC3 fused to GFP (GFP-LC3) (Bampton et al., 2005). Sixteen hours later cells were induced with Dox and assessed 24 hr later for GFP-LC3 localization. In the absence of Dox, as expected, GFP-LC3 was diffuse within the cytoplasm with occasional puncta representing the basal level of autophagosomes within the cell (Figure 5E). Upon DRAM and p53 induction, similar to what is seen

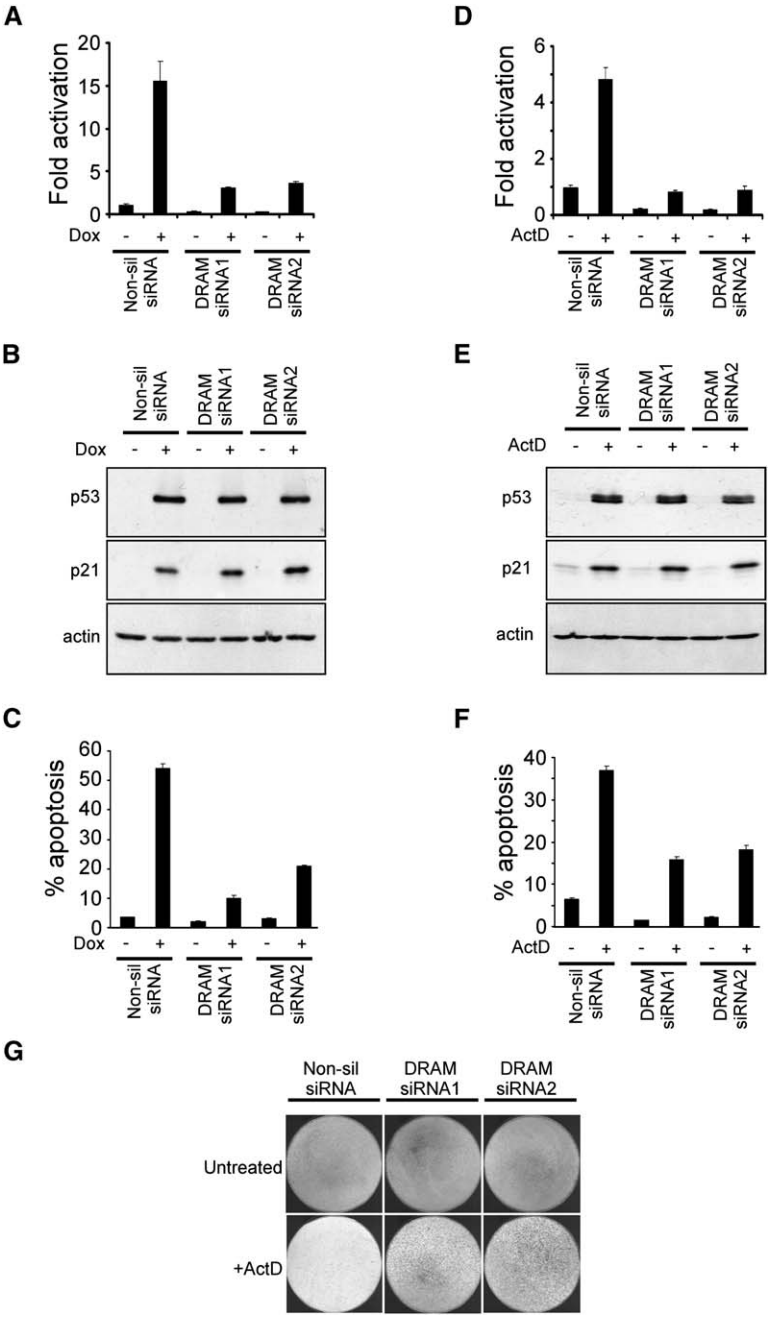

Figure 3. DRAM Is an Important Component of p53-Induced Apoptosis

(A–F) TetOn-p53 cells (A–C) or RKO cells (D–F) were transfected with *DRAM* siRNAs or a nonsilencing siRNA. Forty-eight hours after transfection the cells were treated for a further 24 hr with Dox (A and B) or 1 nM ActD (D and E). *DRAM* mRNA levels were analyzed by qPCR (A and D) and presented as mean fold activation ± SEM. Protein levels of p53 and p21 were analyzed by Western blotting (B and E). Cell death was assessed by flow cytometry after 48 hr Dox or ActD treatment (C and F). The percentage of cells with a sub-G1 DNA content was taken as a measure of cell death. Data are presented as mean % apoptosis ± SEM.

(G) RKO cells were transfected with *DRAM* siRNAs or a nonsilencing siRNA. After 48 hr the cells were treated with ActD for 24 hr. The cells were then replated in fresh media and assessed for clonogenic survival.

following amino acid and serum starvation (a known inducer of autophagy) (Klionsky and Emr, 2000), a marked increase in the presence of GFP-LC3 puncta was observed, indicating a clear role for DRAM and p53 in the regulation of autophagy (treatment of parental Saos-2 with Dox as control showed no increase in puncta) (Figure 5E). Moreover, when quantified, approximately 40% of the cells showed considerable GFP-LC3 puncta following p53 and DRAM activation (Figure 5F). We examined next if the ability of p53 to regulate autophagy was dependent on DRAM. TetOn-p53 cells were transfected with DRAM or nonsilencing siRNAs. Cells were then infected with GFP-LC3 and p53 induced. This revealed that while GFP-LC3 puncta were clearly seen following p53 activation in cells treated with nonsilencing siRNA, those treated with DRAM siRNA displayed a reduced number of GFP-LC3 puncta, indicating an essential role of DRAM in the ability of p53 to modulate autophagy (Figure 5G). Moreover, since LC3-II has a faster electrophoretic mobility than LC3-I, analysis by Western blotting confirmed again that these changes were occurring in the whole population of cells (Figure 5H).

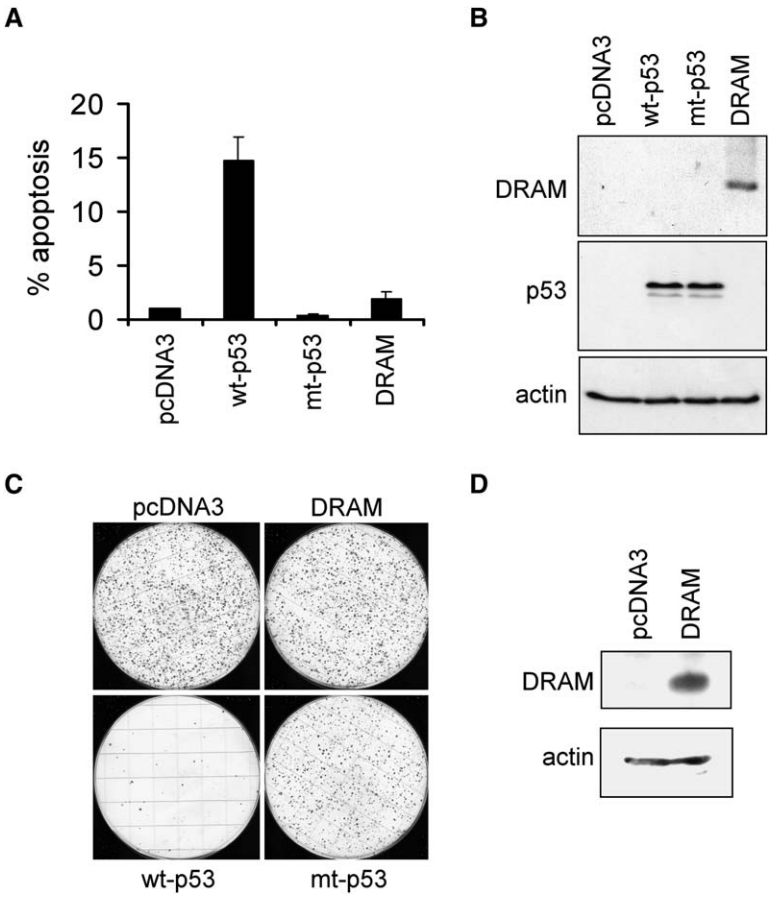

Figure 4. DRAM Is Not Sufficient for Cell Death

(A and B) Saos-2 cells were transfected with plasmids encoding wild-type *p53*, mutant *p53*, or *DRAM* and cell death assayed in the short term (after 24 hr) by flow cytometry (A). Data are presented as mean % apoptosis ± SEM. Protein levels of myc-tagged DRAM and p53 were analyzed by Western blotting (B). (C and D) The longer term effects of DRAM expression were assayed in relation to clonogenic survival. Saos-2 cells were transfected with plasmids encoding wild-type *p53*, mutant *p53*, or *DRAM*. Following selection, cells were replated and assessed for clonogenic survival (C) and long-term expression of myc-tagged DRAM was analyzed by Western blotting (D).

p53 Induces Autophagy in a DRAM-Dependent Manner

Although our data clearly show that p53 and DRAM can regulate autophagy, the appearance of autophagosomes does not necessarily indicate induction of autophagy. Since autophagosomes are only transient in this process, being subsequently turned over in autolysosomes, the accumulation of LC3-II puncta could either represent an actual increased induction of autophagy or a decrease in autophagosome turnover. In fact, agents such as Bafilomycin A1 that block turnover are known to cause autophagosome accumulation (Figure S4) (Boya et al., 2005). Since autophagy is a mechanism by which long-lived proteins are degraded, analysis of turnover of radiolabeled, long-lived proteins is one way to resolve this issue. Following p53 activation we observed a marked increase in the rate of degradation of long-lived proteins, indicating that p53 induces autophagy (Figure 6A). Moreover, this effect was inhibited not only, as would be expected, by knockdown of the essential autophagy gene *ATG5*, using a previously described siRNA, but also by knockdown of DRAM (Figures 6A and 6B) (Boya et al., 2005). This therefore confirms that p53 induces autophagy in a DRAM-dependent manner.

Our results would predict that if DRAM is required for p53 to induce autophagy and cell death, then induction of autophagy may be required for p53-induced apoptotic death. To test this we analyzed p53-induced death following ATG5 knockdown. As this is an undescribed role for ATG5, we used two siRNAs to discount off-target effects. Both siRNAs effectively downregulated ATG5 (Figure 6B) and also caused a dramatic decrease in cell death following treatment of TetOn-p53 cells and RKO cells with Dox and ActD, respectively (Figures 6C and 6D). Furthermore, when DRAM and ATG5 siRNAs were administered together this did not cause any greater reduction in death than DRAM siRNA alone following p53 activation in TetOn-p53 cells (despite knockdown efficiency similar to that seen in single transfections; data not shown). These data therefore indicate that DRAM and ATG5 potentially both control death through their involvement in autophagy (Figure 6E).

Downregulation of DRAM in Human Cancer

Since our data are consistent with a potential tumor-suppressive function for DRAM, we assessed if DRAM is perturbed in human cancer. In the first instance, we analyzed *DRAM* expression by qPCR in cultures of primary normal keratinocytes and in a panel of oral tumor cell lines. This revealed that *DRAM* was significantly downregulated in the tumor lines with the average expression level in these lines being approximately half of that in normal cells ($p < 0.001$) (Figure 7A).

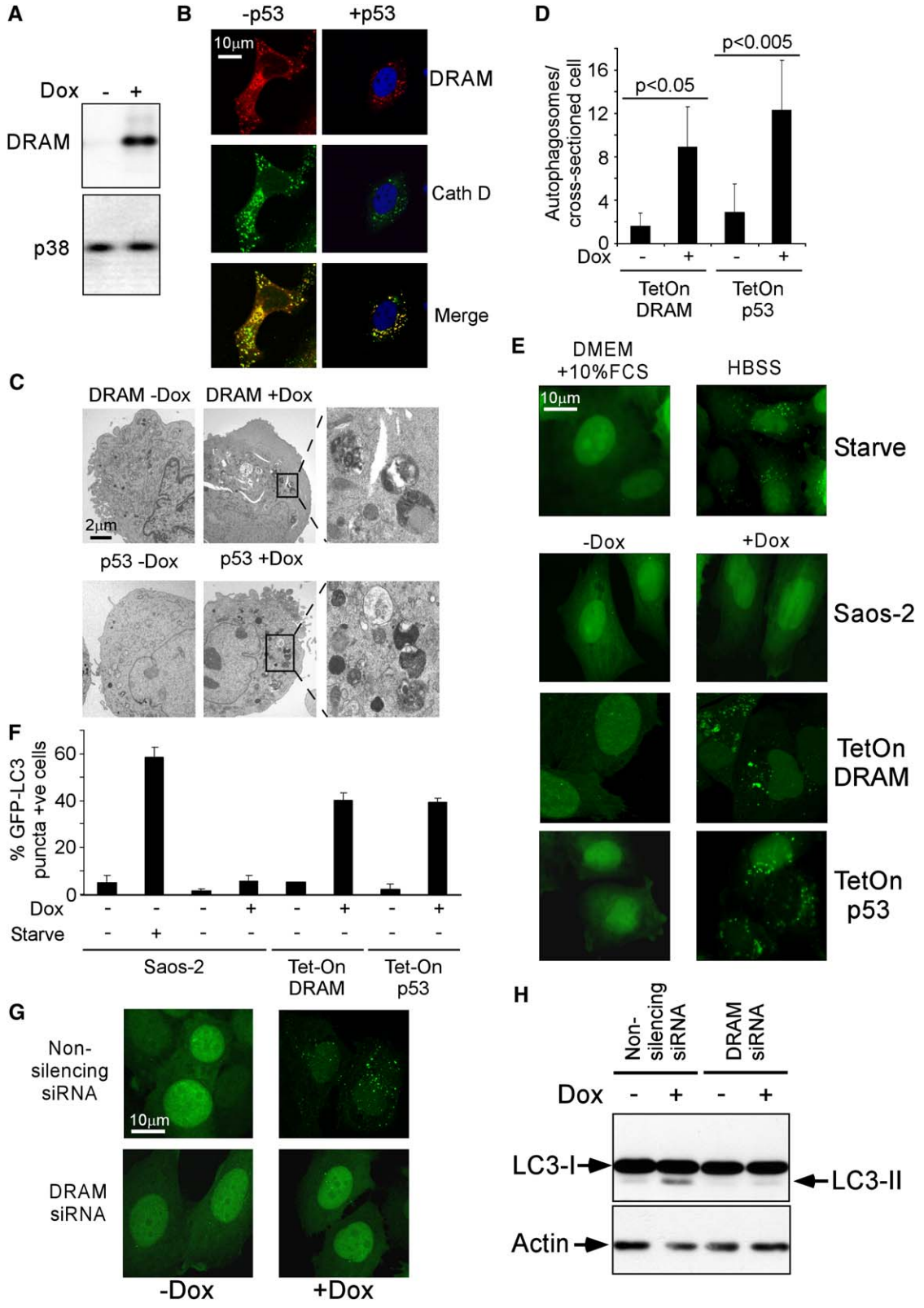

Figure 5. DRAM Is a Lysosomal Protein that Regulates Autophagy

(A) TetOn-DRAM cells were treated with Dox for 24 hr and induction of myc-tagged DRAM was determined by Western blotting.

In the cell lines analyzed, the *DRAM* mRNA was downregulated instead of being completely lost. In many cancers, tumor suppressor gene function can be inactivated without loss or mutation of the gene through epigenetic silencing of gene expression. The most common form of silencing involves cytosine methylation at CpG islands within gene promoters, which is responsible for silencing of tumor suppressors such as those encoded by the *INK4A* and *MLH1* loci (Herman and Baylin, 2003). If methylation is involved in the decreased expression of a particular gene—either directly or through silencing of a positive regulator of the gene—expression of the mRNA can often be enhanced by treatment with the demethylating agent, 5-aza-2′-deoxycytidine (5′-aza) (Herman and Baylin, 2003). Since the *DRAM* gene contains a CpG island in its promoter, we tested if 5′-aza could increase the levels of *DRAM* mRNA in tumor line 8. Indeed, consistent with a role for methylation in loss of *DRAM* expression, 5′-aza resulted in a considerable increase in *DRAM* mRNA levels (Figure 7B). Similar results were also seen in tumor line 13 (data not shown).

Together, these observations imply that *DRAM* expression may be subject to methylation-dependent transcriptional silencing in some human cancers. To address this issue further, we performed methylation-specific PCR (MSP) analysis on two areas of the *DRAM* CpG island in a panel of primary squamous tumors (from head and neck and vulva) and tumors of the breast. We detected no evidence of aberrant CpG methylation at either location in 48 cases of breast cancer (data not shown). However, consistent with our analysis of *DRAM* expression in oral tumor cell lines (Figure 7A), methylation was clearly and reproducibly detected in 16/116 squamous tumors (Table S1).

cDNA of adequate quality was available for five squamous tumors with patient-matched normal tissue, and we therefore performed RT-PCR analysis to assess *DRAM* expression in these cases. Steady-state levels of *DRAM* mRNA were reduced in three of the five cancers relative to matched normal tissue (Figure 7C). These included one case shown to have methylation in the *DRAM* CpG island but also two cases in which no methylation was detected with either primer pair (Figure 7D). These results imply that downregulation of *DRAM* mRNA in squamous cancers occurs both by direct hypermethylation within the CpG island of the gene and also by other, as yet unidentified, mechanisms, perhaps involving epigenetic modification in other genes upstream of *DRAM*. In light of this, we went back to analyze the level of *DRAM* expression by qPCR in our panel of tumors and, although matched normal tissue was not available for all tumors, the levels of *DRAM* expression were assessed by comparison to the average level of expression in available samples of normal tissue (n = 12). This revealed that, consistent with our small analysis of patient-matched normal and tumor samples (Figure 7C and 7D), although all tumors with *DRAM* promoter methylation had downregulation of *DRAM* mRNA, *DRAM* expression was also decreased in some tumors where methylation was not evident. Altogether, 57/116 tumors showed decreased *DRAM* expression (Table S1).

Since *DRAM* is a p53 target gene, we examined whether *DRAM* expression was related to the p53 status of the squamous cancers. Of the tumors containing wild-type p53 (and which were HPV-negative), 79% (42/53) exhibited *DRAM* downregulation. Reciprocally, of those with mutant *p53* (or which were HPV-positive), only 23% (15/63) had downregulated *DRAM*. This difference was highly significant ($Chi^2 = 35.4$, $p < 0.001$). This correlation was even more striking when lesions of vulval origin were analyzed alone—only 18% (3/17) had mutant *p53* (or were HPV-positive) and *DRAM* downregulation, whereas 87% (13/15) had wild-type *p53* and *DRAM* downregulation ($Chi^2 = 21.0$, $p < 0.001$) (Table S1). Together these results suggest that downregulation of *DRAM* occurs preferentially in cancers lacking other mechanisms for inactivation of p53. This relationship was, however, not completely reciprocal with some tumors having decreased *DRAM* levels and mutant *p53*, indicating therefore that that there may be additional selective pressures to inactivate DRAM over and above its role in p53-mediated tumor suppression.

DISCUSSION

DRAM Is a Novel Mediator of p53-Induced Autophagy

Autophagy is an evolutionarily conserved process that was first defined genetically in yeast (Klionsky and Emr, 2000). DRAM is also evolutionarily conserved with orthologs in

(B) DRAM colocalizes with lysosomes. Colocalization (yellow) of myc-tagged DRAM (red) and the lysosomal protein cathepsin D (green) was assayed by confocal microscopy either in the absence or presence of adenoviral p53 (blue).

(C–F) DRAM and p53 expression induce the formation of autophagosomes. (C) representative transmission electron micrographs of TetOn-DRAM and TetOn-p53 cells with and without 24 hr Dox treatment. N = nucleus. (D) Quantification of autophagosomes per cross-sectioned cell presented as mean number of autophagasomes per cell ± SEM. (E) TetOn-DRAM, TetOn-p53, or Saos-2 cells were infected with an adenovirus expressing GFP-LC3 fusion. Where indicated, cells were treated with Dox for 24 hr or amino acid starved in HBSS for 8 hr, fixed, and assayed for the appearance of autophagosomes by confocal microscopy. (F) Quantification of autophagasome formation. Cells with eight or more GFP-LC3 puncta were considered to have accumulated autophagosomes. Data are presented as mean % GFP-LC3-positive cells ± SEM in three independent experiments. In each treatment at least 50 cells were analyzed.

(G and H) Autophagosome formation by p53 is dependent on DRAM. After 48 hr DRAM or nonsilencing siRNA-transfected TetOn-p53 cells were infected with GFP-LC3 expressing adenovirus followed by treatment with Dox for 24 hr. The effects of DRAM knockdown on the ability of p53 to cause accumulation of autophagosomes was determined by confocal microscopy (G), and the relative level of GFP-LC3-I/GFP-LC3-II in the whole population was determined by Western blotting (H). The Western blot is representative of what was seen in five independent experiments.

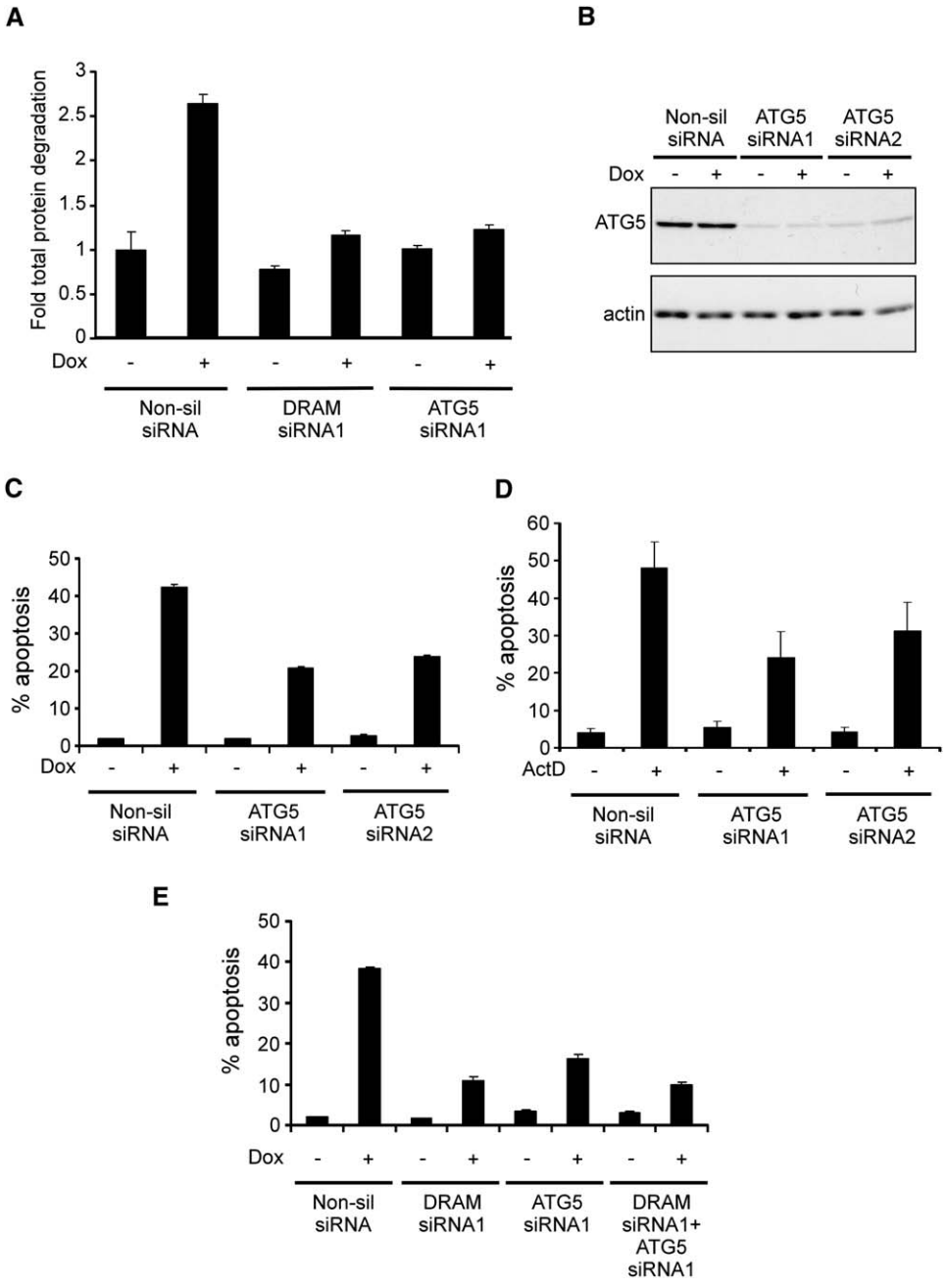

Figure 6. DRAM-Dependent Induction of Autophagy Is Required for p53-Mediated Apoptosis

(A) p53 induces long-lived protein degradation through DRAM. The rate of long-lived protein turnover was measured over 4 hr in TetOn-p53 cells following transfection of nonsilencing, DRAM, or ATG5 siRNAs and induction of p53 with Dox for 24 hr. Data are presented as mean fold protein degradation ± SEM.

(B–D) Autophagy is required for p53-induced apoptosis. TetOn-p53 and RKO cells were transfected with nonsilencing or ATG5 siRNAs. Relative levels of ATG5 protein in TetOn-p53 cells were assessed by Western blotting (B) and levels of apoptosis analyzed by flow cytometry following 48 hr treatment with Dox (C) and ActD (D).

(E) Collective DRAM and ATG5 knockdown does not produce an additive effect. TetOn-p53 cells were transfected with nonsilencing ATG5 and/or DRAM siRNA and cell death measures flow cytometry after Dox treatment (48 hr).

(C)–(E) are presented as mean % apoptosis ± SEM.

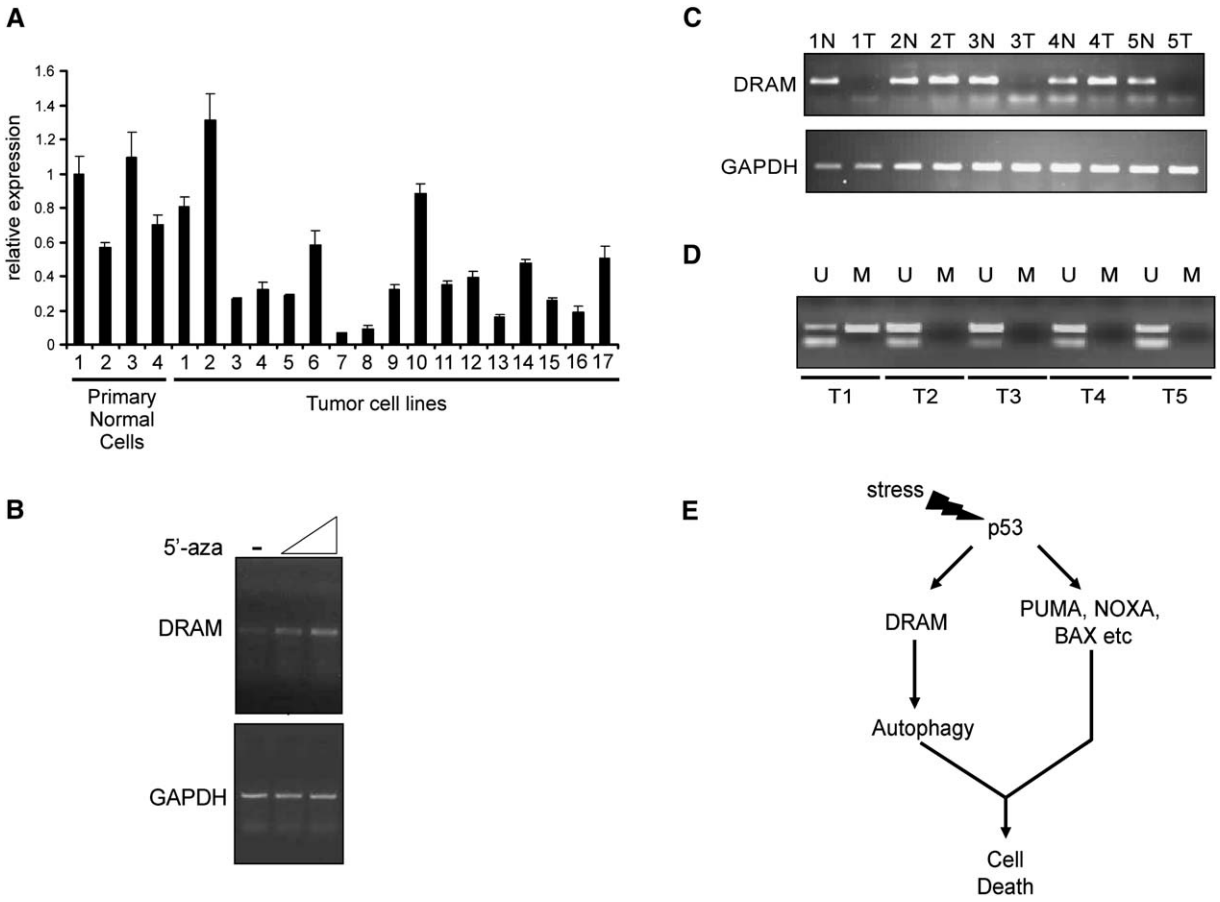

Figure 7. DRAM Is Downregulated in Human Cancer

(A) The expression of *DRAM* mRNA in oral tumor lines or normal keratinocytes was determined by qPCR and presented as mean relative expression ± SEM.

(B) DRAM is subject to epigenetic silencing. *DRAM* mRNA expression was analyzed in tumor line 8 by qPCR following 7 days of treatment with 1 and 2 μM 5-aza-2′-deoxycytidine (5′-aza).

(C and D) Downregulation of *DRAM* mRNA occurs in tumors both with and without aberrant methylation of the *DRAM* CpG island. (C) RT-PCR analysis of *DRAM* expression in squamous cell carcinomas of the head and neck (SCC). The figure shows patient-matched pairs of normal (N) and tumor (T) tissue. (D) MSP analysis of methylation in the *DRAM* CpG island in the same five SCC analyzed for *DRAM* expression.

(E) Model. Stress-induced induction of *DRAM* through p53 induces autophagy to co-operate with one or more other p53-dependent apoptotic signals to invoke a full cell-death response.

a number of species. Multiple stimuli induce autophagy in mammalian cells, but little is known about the regulatory pathways downstream of these stimuli. The discovery of DRAM heralds a new regulator of autophagy involved in the induction of autophagy by p53 in response to genotoxic stress.

The Relationship of DRAM and Autophagy to Apoptotic Cell Death

The identification of *DRAM* as a p53 target mediating induction of autophagy allows exploration of the role of autophagy in apoptosis. Previously, it has been suggested that autophagy may be activated by pro-death stimuli to effect a caspase-independent cell death (Shimizu et al., 2004; Yu et al., 2004). However, it was unclear that autophagy had any role in classical apoptosis. We find that DRAM cannot induce apoptosis itself, but DRAM nevertheless is a critical component of p53′s apoptotic response. Our data therefore do not indicate that p53 induces cell death solely by inducing autophagy through DRAM. This supports a model in which p53 both activates *DRAM*, as well as one or more other proapoptotic genes, and that the signaling pathways regulated by these genes converge, at a yet unidentified point, to promote a full cell-death response (Figure 7E). In this regard, our initial analysis indicates that while inhibition of *DRAM* expression does not affect the activation of p53 targets including *p21* and *PUMA* (Figures 3B, 3E, and S3), it does impede the release of cytochrome *c* from mitochondria (Figure S6). Further studies are, however, required to determine if this is definitively the point at which these p53 pathways converge, and how, specifically, DRAM and potentially

DRAM-induced autophagy contribute to apoptosis. We believe that DRAM contributes to apoptosis through its role in autophagy, as knockdown of ATG5 reveals that autophagy is required for p53's apoptotic response. However, it remains possible that the apoptotic and autophagic functions of DRAM are separable and act in parallel. Future mutational analysis of DRAM may resolve this issue.

Although ATG5 and DRAM are both clearly required for p53-induced death, a further question is whether inhibition of DRAM or autophagy per se through ATG5 knockdown are always equivalent events. It is likely that specific signaling pathways activate autophagy to effect different outcomes in response to specific stimuli. We have shown that DRAM is critical for induction of autophagy in the specific context of p53 activation, whereas ATG5 is predicted to be required for autophagy in all contexts including basal autophagy (Kuma et al., 2004; Mizushima et al., 2001). Future work will address if other pathways involve DRAM. However, with respect to this, while we found that DRAM knockdown can confer a long-term survival advantage to cells treated transiently with ActD, ATG5 does not (data not shown). In fact, chronic knockdown of ATG5 was detrimental to the clonogenic potential of the cells even in the absence of ActD (data not shown). This indicates therefore that the cells have a dependency on ATG5-mediated autophagy for maximal viability long-term, but consistent with a context-specific role for DRAM-induced autophagy downstream of p53, this role of ATG5 is not downstream of DRAM function. Indeed, the chronic knockdown of DRAM is not detrimental to clonogenic potential in the absence of ActD (data not shown). One might speculate therefore, as is emerging from other context-specific autophagy studies, that DRAM-induced autophagy turns over a specific spectrum of proteins, different from other autophagy-inducing signals that also act through ATG5. Perhaps identifying these proteins will help elucidate the mechanism by which DRAM-induced autophagy contributes to cytochrome *c* release and cell death downstream of p53.

Downregulation of DRAM in Cancer

We show here that expression of *DRAM* is downregulated in a subset of epithelial cancers, and we present evidence that downregulation occurs both via direct hypermethylation within the *DRAM* CpG island and by other mechanisms that do not directly target the *DRAM* CpG island. This is not the first report, however, of a regulator of autophagy being perturbed in human cancer. *Beclin1*, which has been shown experimentally to be a haploinsufficient tumor suppressor, is mono-allelically deleted in some breast, ovarian, and prostate tumors (Aita et al., 1999; Liang et al., 1999). As with ATG5, one would predict that Beclin1 would affect autophagy downstream of multiple signals and there may be different selective pressures to downregulate DRAM or Beclin1 in different tumor settings. In this regard, it is interesting to note that we saw no methylation of the *DRAM* gene and limited downregulation of *DRAM* mRNA in breast cancers (data not shown) —a tumor type known to exhibit Beclin1 downregulation.

The relationship of *DRAM* mRNA expression to *p53* status is also provocative. Although a number of p53 target genes, for example *Bax*, *Apaf-1*, and *14-3-3σ*, have been shown to be inactivated in human cancer, none have shown such a reciprocal relationship to mutation of p53 as has *DRAM* in squamous tumors (Gasco et al., 2002; Rampino et al., 1997; Soengas et al., 2001). In therapeutic terms this is potentially very exciting. Due to the critical role of DRAM in p53-induced death, it is possible that there are tumors retaining wild-type p53 that are chemoresistant because of the loss of DRAM. The combination therefore of standard chemotherapeutic agents that stimulate p53 with those that mimic DRAM's role in autophagy may well lead to enhanced tumor cell death.

Overall, the discovery of DRAM reveals a novel link in the pathway by which p53 modulates autophagy and suggests that induction of autophagy by p53 via DRAM contributes to apoptotic cell death. This function of DRAM may account for our observed tumor expression profile in squamous cancers indicative of a tumor suppressor. Our elucidation and further investigation of DRAM function may therefore aid our understanding of tumor suppression and lead to the development of novel agents for cancer therapy.

EXPERIMENTAL PROCEDURES

Please see Supplemental Experimental Procedures for information on cell line generation, plasmids, sequences for ChIP, microscopy, microarrays, sequence analysis, and statistical tests.

Cell Culture and Transfections

Saos-2, RKO, and RKO-pRS-Scr, RKO-pRS-p53, TetOn-p53, TetOn-p53-175H, and TetOn-DRAM cells were grown in DMEM supplemented with 10% FCS and were transfected with $CaPO_4$. TetOn lines were induced with 1 μg/ml Dox (Sigma). For starvation conditions, cells were incubated in Hanks's Balanced Salt Solution (Sigma) supplemented with 10 mM Hepes. Saos-2 and RKO cells are available from ATCC. TetOn-p53 and TetOn-p53-175H cells have been previously described (Ryan et al., 2000). Primary keratinocyte cultures and oral tumor lines have been previously described—details can be obtained on request (Edington et al., 1995; McGregor et al., 2002).

RNAi

siRNA oligos (Dharmacon) targeting the following mRNAs were: DRAM, CCACGATGTATACAAGATA (1) and CCACAGAAATCAATGGTGA (2). ATG5, GCAACTCTGGATGGGATTG (1) and CATCTGAGCTACCCGGATA (2). The nonsilencing sequence was TAAGGCTATGAAGAGATAC. siRNAs were transfected using oligofectamine reagent (Invitrogen).

Cell-Death Assays

Total populations of cells were processed for flow cytometric analysis (FACScan, Becton Dickinson) as previously described (Ryan et al., 2000). The percentage of cells with a sub-G1 DNA content was taken as a measure of apoptotic rate. Clonogenicity assays were performed on cells transfected with the indicated plasmids (Saos-2) or siRNA (RKO). Saos-2 cells were selected with 600 μg/ml G418 (Invitrogen) and after 2 weeks were stained with Giemsa (Sigma).

Protein Degradation

After 24 hr, siRNA transfected TetOn-p53 cells were labeled for 6 hr with L-[^{35}S]Met/Cys (5 μCi/ml) (Amersham), washed three times in PBS, then incubated for 16 hr in DMEM supplemented with 2 mM unlabeled L-Met/Cys plus Dox where indicated. The degradation period was started by washing the cells again and replacing with fresh medium. After 4 hr the levels of degraded protein were calculated as previously described (Boya et al., 2005).

Immunoblotting

Cells were lysed in RIPA buffer and proteins quantified using a BCA assay (Sigma). Equal amounts were separated on SDS-PAGE gels. Membranes were probed with antibodies against: p53 (DO-1, Pharmingen), p38 (#9212, Cell Signaling Technology), myc (4A6, Upstate), actin (clone 1A4, Sigma), p21 (sc-397G, Santa Cruz), GFP (Roche), P-S6K (T421/S424) (Cell Signaling), and ATG5 (a kind gift of Noboru Mizushima) (Mizushima et al., 2001). Proteins were detected by ECL (Amersham).

Luciferase Reporter Assays

Saos-2 cells were transfected with 5 μg of each reporter plasmid, 5 μg of pJ3ΩβgaI, and 0.5 μg of either pcDNA3-p53 or empty vector. Twenty-four hours later, cells were lysed in luciferase lysis buffer (Promega) according to the manufacturer's instructions. Samples were assayed for luciferase activity and values obtained were normalized for transfection efficiency following assay for β-gal activity.

Supplemental Data

Supplemental Data include five figures, one table, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/126/1/121/DC1/.

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